EXHIBIT 7

HUMAN GENE TRANSFER/THERAPY PROTOCOL

9206-019

Oldfield, Edward, National Institutes of Health; *Gene Therapy for the Treatment of Brain Tumors Using Intra-Tumoral Transduction with the Thymidine Kinase Gene and Intravenous Ganciclovir.*

Date of RAC Approval: 6/1/92
Date of NIH Approval: 8/14/92

PROPOSAL FOR A CLINICAL RESEARCH PROJECT

Project Title: Gene Therapy for the Treatment of Brain Tumors Using Intra-Tumoral Transduction with the Thymidine Kinase Gene and Intravenous Ganciclovir.

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Estimated Duration of Study: 3 years.

Number and Kind of Subjects:

	Number	Sex	Age
Normal			
Employee			
Patients	20	M & F	> 18
TOTAL	20		

Gene Therapy for the Treatment of Brain Tumors Using Intra-Tumoral Transduction with the Thymidine Kinase Gene and Intravenous Ganciclovir.

This protocol presents a new therapeutic approach to the treatment of patients with otherwise incurable malignant brain tumors. The strategy involves the preferential introduction of a drug-susceptibility gene into proliferating tumor tissue. This is accomplished by stereotaxic infiltration of the brain tumor with murine cells engineered to produce a retroviral vector which carries the gene for herpes simplex thymidine kinase. The injected vector-producer cells will continuously release HS-tk-carrying virus in the vicinity of the tumor over a period of days. Retroviral vectors will only transfer genes into proliferating cells and, in the brains of these patients, the only mitotically active cells will be the tumors.

The herpes TK gene confirs sensitivity upon the cells expressing the gene to the antiviral drug ganciclovir (GCV). GCV is nontoxic to normal tissues, but will kill cells (eg. tumors) expressing this herpes virus enzyme. Our data also suggest that bystander tumor cells, not expressing HS-tk, may also be killed as a consequence of this process, amplifying the antitumor effects of treatment (by a process not completely understood).

Precis:

Malignant brain tumors are responsible for significant morbidity and mortality in both pediatric and adult populations. These common tumors present an enormous therapeutic challenge due to their poor outcome despite radical surgery, high dose radiotherapy and chemotherapy. Survival of patients from the time of diagnosis is measured in months and recurrence after treatment is associated with a life expectancy of weeks.

In an attempt to improve this grim prognosis of patients with malignant brain tumors (both primary tumors and secondary metastasis from systemic cancer such as melanoma, lung and breast cancer), we have developed a novel approach to the therapy of brain tumors. This approach makes use of recombinant DNA technology to transfer a sensitivity gene into a brain tumor. This is acheived by direct injection of the

tumor with a cell line actively producing a retroviral vector carrying a gene conferring drug sensitivity to the tumor. A retroviral vector is a mouse retrovirus genetically engineered to replace its own genes with a new gene. Such vectors are capable of "infecting" mammalian cells and stably incorporate their new genetic material into the genome of the infected host. The producer cell is an NIH 3T3 cell that has been genetically engineered to continually produce retroviral vectors.The new gene is incorporated into the genome of the tumor cells and expresses the protein which is encoded by the new gene. This protein (the herpes simplex virus enzyme thymidine kinase, HS-tk) sensitizes the tumor cells to an antiviral drug (ganciclovir, GCV) which is a natural substrate for HS-tk. The enzymatic process induced by GCV leads to death of the cell expressing the herpes TK activity, i.e., death of the tumor cells. Since the HS-tk enzyme which is normally present in mammalian cells has very low affinity for GCV, systemic toxicity related to this mechanism is not observed. This type of in vivo gene transfer has several unique features. First, these retroviral-vectors will only integrate and express their genes in cells which are actively synthesizing DNA. Therefore, surrounding non-proliferating normal brain tissue should not acquire the HS-tk gene and will remain insensitive to GCV. Second, all of the transduced tumor cells (and retroviral vector producing cells) will be killed by the host immune response and/or GCV treatment eliminating potential concern about insertional mutagenesis giving rise to malignant cells.

This is the first clinical attempt to treat malignant tumors in human beings by in-vivo genetic manipulation of the tumor's genome.

<u>Overall Study Design</u>

The study was designed to provide information on three aspects of this therapy: 1. Is there significant toxicity associated with our approach, despite the safety which was shown in the various animal studies. 2. Does in-vivo transduction take place in the brain tumor, and if so, what is the extent of transduction, and 3. What is the efficacy (biological effect) of our approach, i.e., the tumoricidal effect in human patients.

Adult patients (>18 years) with brain tumors will be evaluated for the extent and location(s) of their disease. Accordingly, the patients will be divided into surgically accessible and inaccessible groups. In both groups of patients, the tumors will be injected with the HS-tk-producing vector using an MRI guided stereotaxic approach.

Surgically accessible lesions will then be debulked (7 days after stereotaxic injection), and the tumor bed will be infiltrated with the HS-tk vector producer cells. The removed tumor will be evaluated for the efficiency of transduction. GCV will be administered beginning on the fifth postoperative day at 5mg/kg/dose BID for 14 days.

The surgically inaccessible lesions will receive an MRI-guided stereotaxic intra-tumoral injection of the retroviral HS-tk vector-producer cells. 7 days later, the patients will undergo intravenous therapy with GCV at 5mg/kg/dose BID for 14 days.

Patients who will exhibit a partial response to therapy will be considered for repeated treatments (cell injection and GCV administration) based on their clinical status, antibody production to PA317 cells, and the radiographical evaluation of their tumors.

Preliminary Phase: The first stage of the study will be limited to treatment of 3 patients with recurrent malignant brain tumors who are considered to have an especially poor prognosis with a very short life expectancy (less than 2-3 months). These patients will undergo treatment according to the study design proposal and will be particularly observed for the development of a significant toxicity which is unresponsive to available therapeutic measures. If such a toxicity is not observed, the study will continue to include the study population described.

Objectives

1. To determine if intratumoral delivery of a vector-producer cell line into human brain tumors will result in *in vivo* transduction of the brain tumor.
2. To determine if ganciclovir administration to the HS-tk-transduced tumor will result in eradication of the tumor.
3. To evaluate the short and long term consequences of the implantation of xenogeneic vector-producing cells.

I. Introduction

A. Clinical Background:

1. Primary Brain Tumors.

Brain tumors are a major cause of morbidity and mortality in the population. New brain tumors develop in approximately 35,000 adult Americans each year. They comprise the third leading cause of death from cancer in persons 15 to 34 years of age (1). Recent evidence indicates that the prevalence of primary brain tumors is increasing, especially in the elderly (2) .

The astroglial brain tumors, including the highly malignant glioblastoma multiforme (GBM), are the most common primary brain tumors. Despite aggressive therapy which includes surgical removal of the tumor and post-operative high dose radiation, the prognosis of patients with GBM is very grim with a median survival of 9 to 10 months (3). Although controversial, it appears that neither the quality nor time of survival is significantly improved when chemotherapy is added to surgery and radiation (4). When glioblastoma multiforme recurs, there is 100% mortality within weeks to a few months. In one study, a mean survival of only 36 weeks was found in patients with recurrent GBM who underwent a second operation. Unfortunately, a reasonable quality of life in those patients was limited to 10 weeks following the diagnosis of recurrent GBM (5).

2. Cerebral Metastases.

Cerebral metastases are a frequent complication of systemic cancer occurring in 20 to 30 percent of patients with cancer (6), (there are 1.1 million new cases of cancer per year in the U.S.). In 50% of patients, the metastatic disease is localized to the central nervous system (7). A subset of patients may even be cured of their primary cancers only to succumb to the isolated metastatic disease in the brain. Surgery, combined with radiation therapy, is the treatment of choice for a single focus of brain metastasis that is surgically accessible. Median survival using the bimodality therapy (surgery and radiotherapy) reaches 40 weeks. In most patients with metastatic disease to the brain, multiplicity of the lesions, or their inaccessibility, prohibits surgical intervention and limits therapy to radiation alone with a median survival of about 15 weeks (8).

3. The Proposed Treatment of Human Brain Tumors

The central nervous system has several advantages of safety and efficacy for in-vivo gene transfer. First, retroviral vectors only integrate and therefore express vector genes in proliferating cells. In the brain, the tumor is the most mitotically active cell, with only macrophage-derived cells, blood cells and endothelial cells at minimal risk. Therefore, the possibility of specific transduction of the tumor is enhanced. Second, the brain is a partially immunologic privileged site, which should allow a somewhat longer survival of the xenogeneic murine cells in the brain and a greater transduction frequency of the growing tumor. A special feature of human gliomas is their ability to depress local immunity. This is thought to be secondary to down regulation of IL-2 secretion and diminished expression of high affinity IL-2 receptors on T-lymphocytes (9). The murine cells should survive longer allowing for the transduction of greater numbers of tumor cells. However, this period of survival will be limited since all cells that integrate and express HS-tk will be destroyed by the GCV.

B. Clinical Gene Transfer

1. Gene Transfer Methods

There are 3 major methods of gene transfer: 1) chemical (e.g. calcium-phosphate precipitation), 2) physical (e.g. microinjection, electroporation, fusion-liposomes) and 3) viral (e.g. herpes simplex, adenovirus, retrovirus) (10). Physical and chemical methods have poor gene integration efficiency, ranging from 1:1,000 to 1:100,000, making them impractical for most clinical applications. The adenoviruses and herpes simplex viruses have been used to transfer the vector genes into cells where they replicate in an extrachromosomal location. Therefore, adenoviruses and herpes simplex viruses can transfer genes into non-replicating tissues (e.g. lung, brain), unlike the murine retroviruses.

2. Retroviral-mediated Gene Transfer (11).

In contrast to chemical and physical methods, murine retroviral vectors have proven to be extremely efficient for gene transfer into mammalian cells, with efficiencies as high as 90% in cultured murine fibroblast cell lines. Murine retroviral vectors differ from the adenoviruses and herpes simplex viruses in that the retroviruses will only integrate and subsequently express their genes in proliferating tissues (e.g. tumor). This feature of the retroviral vectors may be particularly advantageous in the

brain, where the tumor is the predominant mitotic cell type, maximizing specific transduction of tumor with minimal, or absent, transduction of normal brain. These Moloney murine leukemia virus-based (MoMLV) vectors have been designed to minimize the possibility of recombinantion resulting in regeneration of a replication-competent virus (12, 13).

3. Experience with Retroviral-mediated Gene Transfer Into Humans (Appendix F).

N2/TIL Marking Study

In 1989, the first gene transfer experiment in humans was conducted at the NIH. This study involved the treatment of 10 patients with autologous T-cells (TIL) that have been transduced with a retroviral-vector. The vector used in this experiment was LNL6 (constructed by A. Dusty Miller; supernate produced by Genetic Therapy Inc.) which has the same general structure and safety modifications as the G1TKSVNa vector we propose to use. None of these patients have demonstrated any untoward effects secondary to receiving the genetically-altered cells (14).

Human Gene Therapy for Adenosine Deaminase Deficiency

2 children have been enrolled in this protocol since it opened in September, 1990. 15 intravenous infusions of genetically altered autologous T-cells have been administered. These children had significant immunodeficiency before receiving these genetically-altered cells, but they have substantially improved after infusions of the LASN-modified cells. They have been treated with the LASN vector (constructed by A. Dusty Miller; supernate produced by Genetic Therapy Inc.) which, like LNL6, has the same general structure and safety modifications as the G1TKSVNa vector that we will use. Neither child has demonstrated any evidence of adverse effect due to the genetically altered cells.

Other Human Gene Therapy Experiments

A number of other gene-marking experiments (e.g. transduction of marrow cells in patients undergoing bone marrow transplantation) and therapy experiments (e.g. insertion of cytokine genes into autologous tumor in an effort to vaccinate a patients) are beginning. No untoward side effects related to retroviral mediated gene transfer have been observed in any patient.

II. Pre-clinical Data

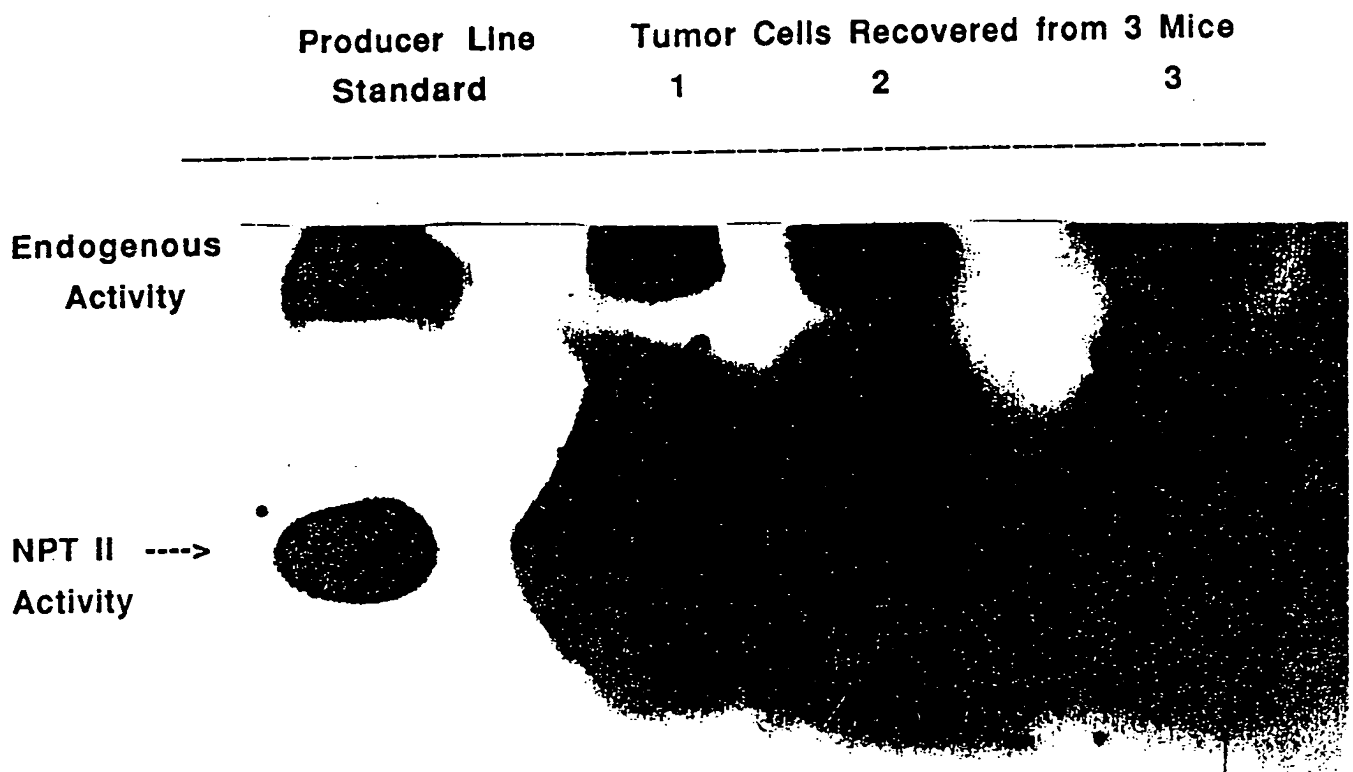
A. In Vivo Transduction with NeoR and β-galactosidase Vectors:

1. In Vivo Transduction of the NeoR Vector (LNL6) into the MCA 205 Murine Fibrosarcoma

Initial studies were performed in mice to determine if tumor cells could be transduced in vivo. The retroviral vector LNL6, which has a titer of $1.0x10^6$ cfu/ml, was utilized for these experiments. LNL6, which contains a NeoR gene promoted by the 5'-LTR, is free of replication-competent retrovirus. NeoR protects mammalian cells from the toxic effects of the neomycin analogue G418. LNL6 vector producer cells or control cells not producing vector but expressing the NeoR gene (LNL6 transduced 3T3 cells) and MCA 205 tumor were mixed in vitro and injected subcutaneously in syngeneic C57BL/6 mice. The mice were inoculated with either $2x10^6$ MCA 205 tumor cells alone (group 1), with $1x10^6$ MCA 205 tumor cells mixed with either $1x10^6$ control NeoR expressing cells (group 2) or with $1x10^6$ NeoR vector producing PA317 cells (group 3). The cells were mixed just prior to subcutaneous injection. The mice were then ear tagged, cages coded and the tumors were measured twice weekly with calipers in 3 dimensions. Tumor size is expressed as a volume (mm^3 : length x width x height).

To minimize the possibility of contamination of the recovered tumor cells with 3T3 and PA317 cells, we waited 4 weeks before excising the growing tumors. The excised tumors were minced and digested into a single cell suspension. Tumor cells from each group were cultured for two weeks. The cultured cells were then placed in a clonogenic assay (table 1). The number of colonies that grew in media without G418 were similar in each group with a cloning efficiency of 15-20%. There were no colonies with resistance to G418 in groups 1 and 2, while group 3 had a mean of 63%±15 (range 55-73) G418-resistant colonies. A functional assay for NPT activity, the enzyme produced by NeoR, was positive for all G418-selected tumors in group 3 (Figure 1).

Figure 1.


Producer Line
Standard
Tumor Cells Recovered from 3 Mice
1
2
3
Endogenous
Activity
NPT II ---->
Activity

In control mice, the injection of LNL6 producer cells alone produces a transient tumor, that is rejected in 7-10 days. Since the evaluation of tumors in the experimental group was performed at 4 weeks, it is very unlikely that these G418 resistant cells are the producer line cells. The lack of G418-resistance in group 2 and the lack of vector production by the recovered G418-selected cells in group 3 suggests that the PA317 producer line is not responsible for the G418-resistance in group 3.

2. In Vivo Transduction of the E. Coli LacZ (β-galactosidase gene) Vector (G1NaSVBG) into the 9L Rat Brain Tumor

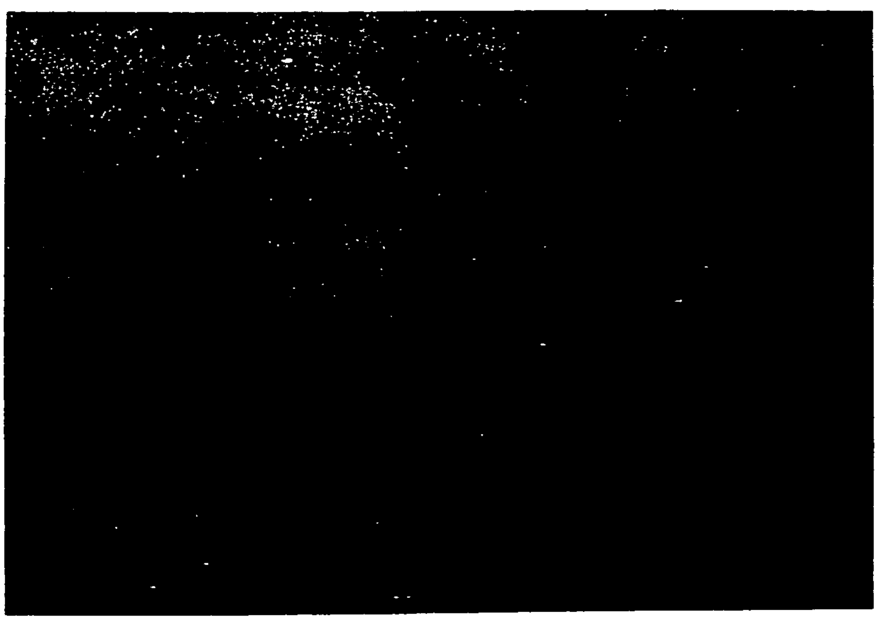
To evaluate the in vivo transduction dynamics within brain tumors, we have used the G1NaSVBG vector. G1NaSVBG (produced by Genetic Therapy Inc., GTI) has a titer of 1-5X10^6 cfu/ml. This vector contains NeoR and the E. Coli derived gene LacZ which encodes for the production of the enzyme β–galactosidase (BAG) (15). The BAG expression can be detected using an X-GAL histochemical stain. Staining the brain with X-Gal turns BAG expressing cells blue. This results when an indolyl is liberated from X-GAL by the action of the BAG enzyme. Subsequent oxidization and self - coupling forms an indigo blue derivative. The vector containing cells can thus be discriminated from unmodified cells and then be enumerated with light microscopy.

Rats were inoculated with 4x10^4 9L gliosarcoma cells into the right cerebral hemisphere using stereotaxic guidance. 7 days later, 3x10^6 G1NaSVBG producer cells were injected into the tumor bearing and non-tumor bearing hemispheres using the same stereotaxic coordinates. 5, 9, and 14 days after injection of the producer line cells, the rats were sacrificed by an intracardiac injection of formaldehyde to fix the brain. The brains were removed and stained with the X-GAL technique. Control rats were injected with a BAG expressing non producer cell line (G1NaSVBG transduced, G418 selected 3T3 non-producer cells). The producer cells and the control G1NaSVBG transduced 3T3 cells were 100% positive by X-Gal staining prior to injection.

In this experiment, we have shown that the injection of producer cells led to transduction of 50-79% of the tumor cells in situ (Table 2). There was no evidence of tumor transduction in the recipient of the non-vector producing group (Figure 2a).

Figures 2b-d. present the microscopic sections taken from the rats on days 5, 9, and 14. The production of the BAG enzyme in tumor cells increased with time as evidenced by the increase in the intensity of the blue stain in each transduced cell. Maximal expression was reached between 7 and 14 days following injection of the G1NaSVBG producer cells. The injected G1NaSVBG producer cells were detected in diminishing numbers up to 14 days when they disappeared from the injection site. There was a clear cut delineation between the transduced tumor cells and normal brain tissue (Figure 2d). Except for the possibility of a rare transduced endothelial cell in the vicinity of the tumor, no evidence of non-tumor brain transduction was evident in either cerebral hemisphere. The most mitotically active endothelial cells in the area of the tumor are likely to be those responding to neovascularization signal from the tumor. Elimination of these endothelial cells with GCV therapy is desirable. The non-producer control cells did not demonstrate evidence of transduction of either tumor cells or normal brain and they were not seen in the tumor or brain after 14 days.

Figure 2.a. - High power magnification of a 9L brain tumor injected with the nonproducer cells. Note absence of X-Gal positive tumor cells.

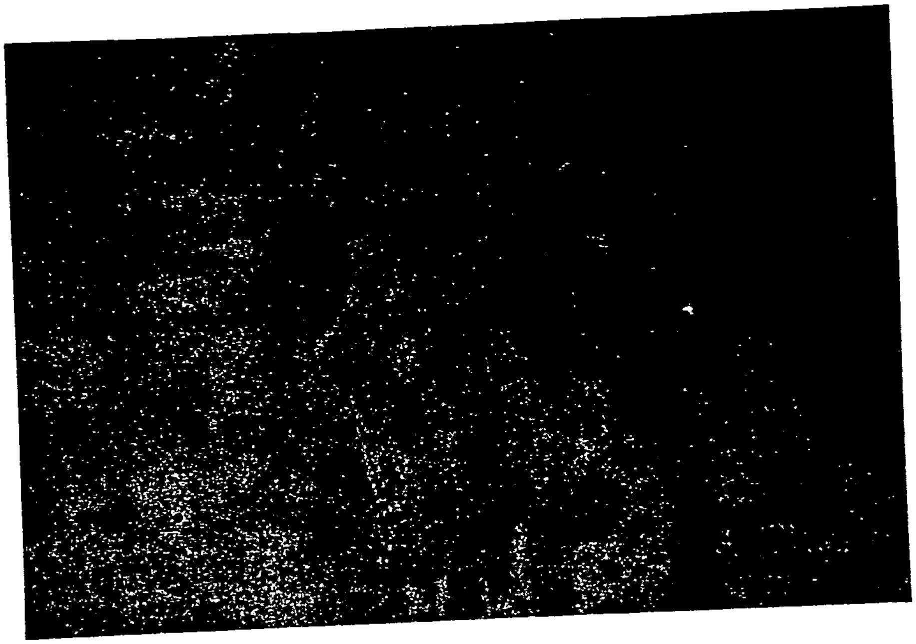


Figures 2 b-d. - Transduced tumor cells.

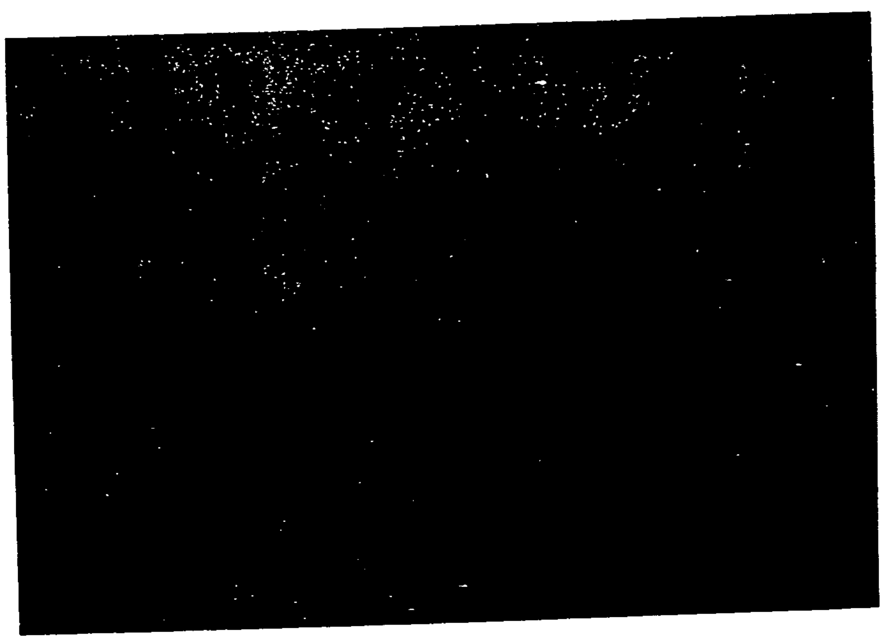
b. Day 5 after injection of the producer cells.
c. Day 9 after injection of the producer cells.
d. Day 14 after injection of the producer cells.

Note the sharp demarcation between the transduced tumor and the surrounding brain (2.d.).

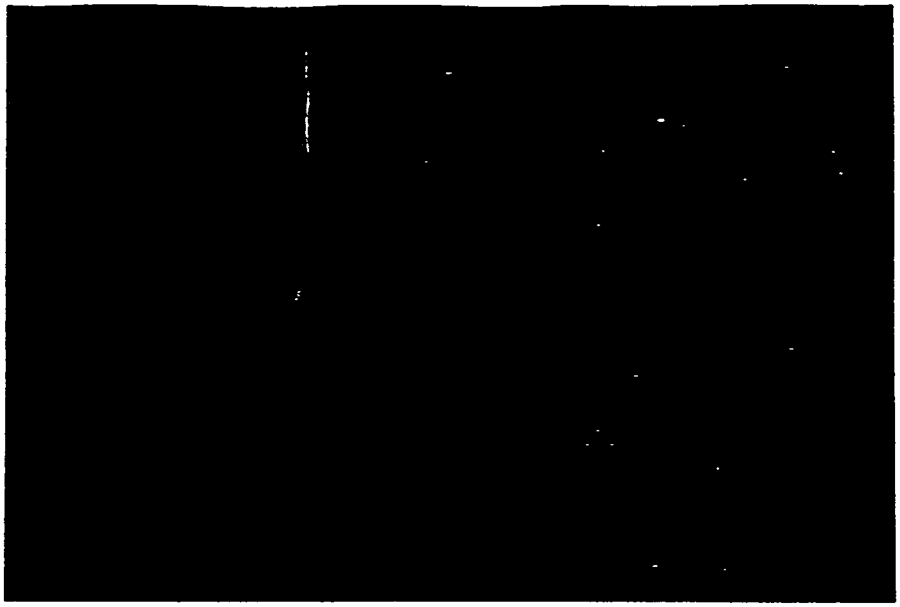
2.b.



2.c.



2.d



This experiment demonstrates that local injection of a vector producer cell line will transduce tumor cells in vivo, that the transduced tumor infiltrates surrounding brain with the non-transduced tumor and that in vivo retroviral-mediated gene transfer does not significantly effect adjacent normal non-proliferating brain tissue.

B. In Vitro GCV Sensitivity of Mammalian Cells +/- Transduction with a HS-tk Vector

The NeoR-HS-tk vector was inserted into murine (NIH 3T3 cells and the MCA 205 fibrosarcoma), rat (9L gliosarcoma), and human (U251 glioblastoma) cell lines in vitro using supernate collected from confluent producer line cells. The transduced cell lines were then selected in G418 for 14 days at 1.0 mg/ml. Only cells expressing a functional NeoR gene are able to survive these G418 culture conditions producing a 100% selected population of vector containing cells.

We then evaluated the sensitivity of the transduced, G418-selected cells compared to the non-transduced parent cell lines (figure 3). In each case, the HS-tk-transduced, G418 selected 3T3 cell lines and HS-tk producer cell line were markedly more sensitive to low concentrations of GCV. Concentrations of 0.5-5 μg/ml were inhibitory to the HS-tk-transduced cells in this assay. In a clonogenic assay, HS-tk

positive cells were completely inhibited at 0.5 μg/ml (Appendix A, Table 1). These findings confirm that HS-tk gene-containing retroviral vectors can effectively transduce murine and rat tumor cells and stably express both the NeoR and HS-tk genes resulting in 100% kill of the transduced cells in vitro when exposed to GCV. All in vivo studies were conducted using G418-selected producer and control non-producer cell lines that had a similar pattern of GCV sensitivity conferred by a transferred HS-tk gene.

Figure 3.a.

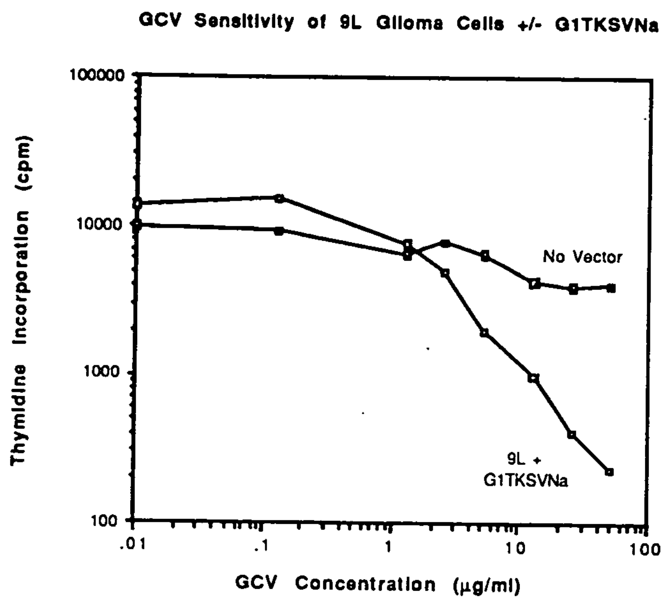


Figure 3.b.

GCV Sensitivity of 205 Fibrosarcoma Cells +/- G1TKSVNa, G1TKSVNa Producer cells and G1TKSVNa Transduced 3T3 cells

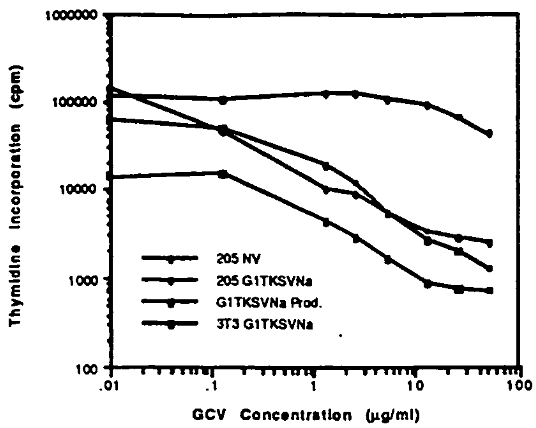
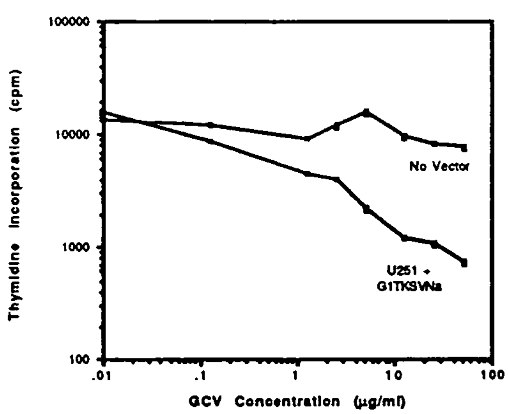


Figure 3.c.

GCV Sensitivity of Human U251 Glioblastoma Cells +/- G1TKSVNa



C. Toxicity studies:

1. Assessment of Transduction of Non Tumor Proliferative Tissues.

Using the β-galactosidase gene as a marker, brain tumors were injected with the G1NaSvBg vector-producer cells. Organs were harvested and stained with X-Gal in order to estimate the frequency of vector expressing cells. Organs evaluated included the heart, lungs, liver, spleen, kidney, and small and large bowel. Organs were examined at various intervals following the intra-tumoral injection of the vector (days 5, 9, and 14 after injection). Organs from rats in which control non-producer BAG cells were injected into their tumors served as controls.

No X-Gal positive cells were seen in the heart and kidney. Occasional X-Gal positive cells were seen in the spleen, liver, and in the lungs, compatible with the distribution of normal macrophages. No difference was observed between the frequency of X-Gal positive cells in these organs between the rats injected with the vector producer cells and those injected with the non-producer cells. In normal bowel, there is a large number of X-Gal positive cells within the villi of both the small and large bowel. Again, no significant difference was observed between the non-producer and the producer treated rats. These findings are consistent with the histological findings suggesting that no significant spread of the vector takes place within normal brain.

2. Assessment of HS-tk-producer toxicity in the peritoneal cavity and in the lung.

We have injected HS-tk vector-producer and control non-producer cells IP into mice. 10 mice received $5x10^6$ HS-tk vector-producer cells and 10 received $5x10^6$ BAG vector-producer cells. The mice were observed for 7 days during which no evidence of toxicity was observed in either group. Ganciclovir was then administered at a dose of 150mg/Kg BID for six days. During and after ganciclovir administration, no toxic side effects were observed. A few mice were sacrificed at different time points. No grossor microscopic pathology was seen in the various organs. The rest of the group is followed up with no long-term signs of toxicity.

In a second group of mice, $5x10^5$ HS-tk vector-producer cells were injected IV via the lateral tail vein to evaluate possible toxicity to the lungs where the cells are trapped. IV injection of $5x10^5$ BAG vector-producer cells were used in control mice. No evidence of toxicity was observed before, during and following ganciclovir administration. Review of micorscpoic slides of the lungs revealed no areas of necrosis or other pathology in comparison to the control group.

3. <u>Toxicity studies of HS-tk vector-producer cells with and without GCV in normal brain.</u>

Rats were inoculated with $3x10^6$ HS-tk vector-producer cells into the deep white matter of the cerebral hemisphere. The contralateral hemisphere served as control with $3x10^6$ BAG-transduced 3T3 cells. Cells were injected in a volume of 50µL. Rats were treated with GCV at a dose of 15mg/Kg BID for 7 days and sacrificed 3 days later for histological evaluation of the brain. The deep injection site was evident in both hemispheres with local changes secondary to the infusion of cells. No difference was seen between the HS-tk vector-producer cell injection site and the BAG-transduced 3T3 cell injection site. Surrounding brain tissue did not show evidence of inflammation or destructive chamges in either group. A repeat experiment with unilateral injection of a mixture of HS-tk-producer cells (90%) and 3T3-BAG (10%) followed by GCV 15mg/Kg BID for 7 days showed similar results. On day 5 following injection of the HS-tk-producer cells, all the rats appeared ill and dehydrated. Similar symptoms were observed when control BAG producer cells alone were injected. All the rats recovered completely after 24 hours of treatment with subcutaneous saline administration. The rats were sacrificed after cessation of GCV. Histology showed mild edema around the injected cells. No viable BAG cells were seen and there was some degree of neutrophilic infiltrate localized to the injection site. In the following repeat toxicity studies, dexamethasone was administered to the rats as an oral dose of 0.5 mg/Kg/day, starting on the third day post injection of the vector-producer cells. In this experiment, as well as in the subsequent experiments in the 9L rat brain tumor model, no evidence of clinical toxicity was observed in the dexamethasone treated rats. It appears that dexamethosone pretreatment diminishes the non-specific symptoms related to the surgery and implantaion of cells in the brain.

In an ongoing toxicity study in Rhesus monkeys, $1x10^7$ HS-tk-producer cells were injected into the right frontal hemisphere of 5 monkeys. All animals were pretreated with high-dose dexamethosone. MRI scans 5 days after inoculation of the cells demonstrated the small (5mm) inoculation site without any evidence of edema or mass effect within the brain. GCV was given to two monkeys on the seventh postoperative day. Again, no neurological ill effects were observed. MRI scan in the monkeys receiving GCV showed the inoculation site without any evidence of edema or mass effect within the brain. Additional data is pending from histological assessment of the brains and organs of three monkeys who will be sacrificed at the termination of the

experiment. The remaining 2 monkeys will remain alive for long-term neurological assessment and virologic follow-up.

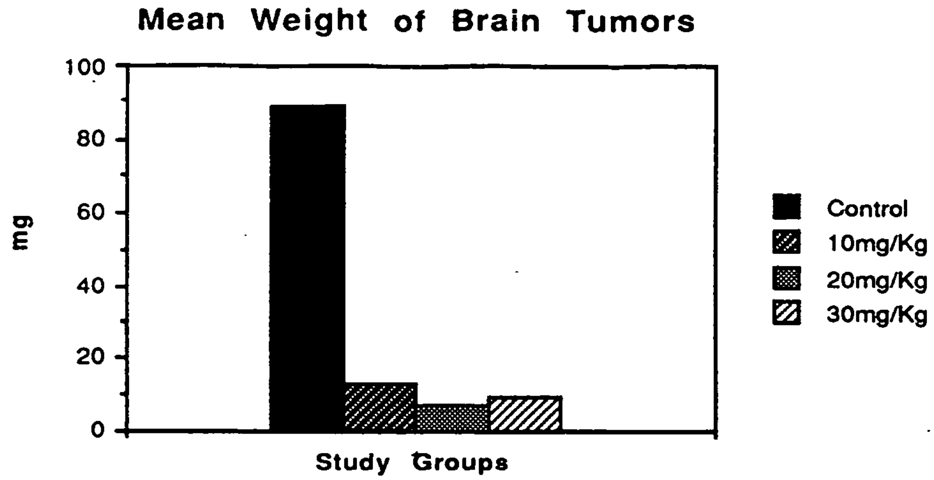
D. Optimization Studies:

1. Ganciclovir Dose Optimization

The optimization of ganciclovir dosage was evaluated in the rat 9L brain tumor model. The purpose of the experiment was to determine the minimal dose of GCV necessary to completely eradicate the tumor. 9L cells, pre-transduced with the HS-tk gene and selected in 1.0mg/ml G418, were used for tumor inoculation. Ganciclovir was given IP at doses of 10, 20, and 30 mg/Kg/day for 6 days.

The animals were sacrificed 3 days following cessation of ganciclovir. In vivo fixation of the brain was achieved with intracardiac infusion of 4% formaldehyde solution. The brains were removed and with the use of the surgical microscope, the tumors were dissected free of the surrounding brain and weighed. Tumors in the ganciclovir-treated groups shrank to a mean weight of less than 10% of the tumors in the untreated control rats and were composed of necrotic non-viable cells. There was no significant difference between the three GCV dosages (Figure 4). Thus, tumor regression was obtained with GCV doses routinely applied to man.

Figure 4.

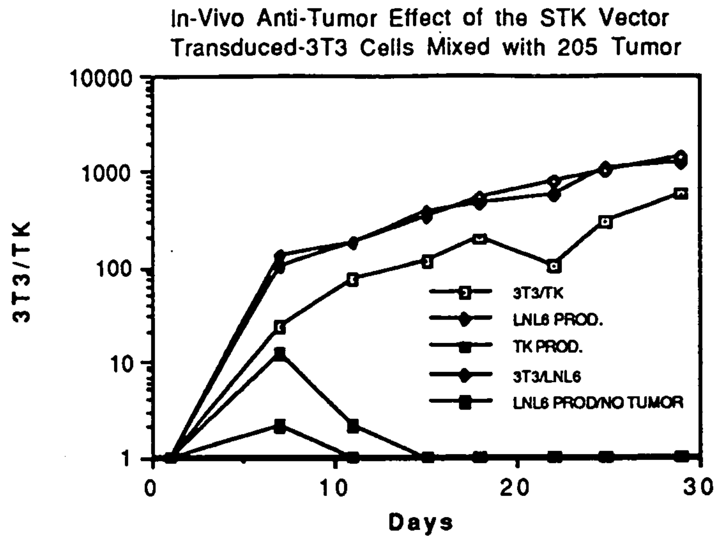


E. <u>In Vivo Transduction with HS-tk Vectors in Mice and Rats:</u>

1. Anti-tumor Effect of In Situ Injection with an HS-tk Vector Producer Cell Line on the In Vivo Growth of the MCA 205 Tumor in Mice

Initially, in order to determine if this in vivo gene transfer technique can promote an increased anti-tumor effect, we used the STK producer cell line. STK contains a NeoR gene promoted by the 5'-LTR and an SV40 promoted HS-tk gene. Figure 5 depicts the effect of GCV on tumor growth in vivo in mice that were injected with $2x10^6$ MCA 205 tumor cells alone (group 1), $1x10^6$ MCA 205 tumor cells plus $1x10^6$ control non-producer HS-tk transduced 3T3 cells (3T3/STK; group 2) or $1x10^6$ MCA 205 tumor cells plus $1x10^6$ PA317 HS-tk vector producer cells (PA317/STK; group 3). The mice were ear tagged, cages coded and the tumors were measured twice weekly with a calipers in 3 dimensions. Tumor size is expressed as a volume (length x width x height). GCV treatment was initiated 4 days after injection of the cells and continued twice daily for 12 doses of 150mg/kg/dose.

Figure 5.



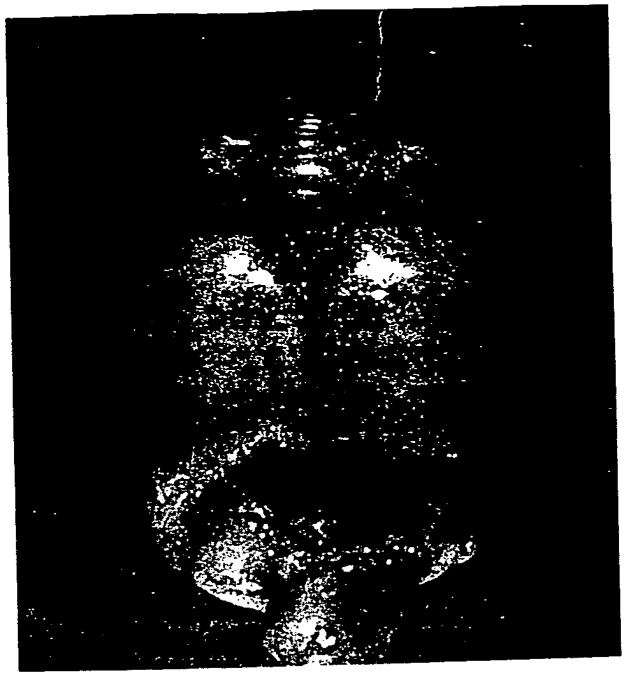
Tumors in groups 1 and 2 grew well regardless of GCV treatment. However, in group 3, if the mice were treated with GCV there was complete tumor elimination. Non-GCV treated tumor cells grew unabated as tumors in groups 1 and 2. The major difference between groups 2 and 3 is the production of vector since the *in vitro* sensitivity of 3T3/STK and the PA317/STK Producer to GCV is not significantly different. In no experiment did GCV alone demonstrate any significant anti-tumor effect on non-HS-tk-transduced tumors. Injection of the LNL6 producer cells result in transient growth and then elimination by day 15 in 5/5 mice. Therefore, these findings suggest that the transfer of a herpes TK gene into tumor in vivo can result in more efficient eradication of MCA 205 tumor than HS-tk-transduced non-producer cells.

2. Anti-tumor Effect of In Situ Injection with an HS-tk Vector Producer Cell Line on the In Vivo Growth of the 9L Gliosarcoma in Rats

9L is a rat gliosarcoma cell line derived from the Fisher 344 strain. This brain tumor model has been well characterized (16). Injection of $4x10^4$ 9L tumor cells into the the cerebral white matter of a rat results in 100% lethality by 4 weeks. Therefore, we have used this brain tumor model to evaluate in vivo HS-tk transduction and the subsequent anti-tumor response to GCV treatment .

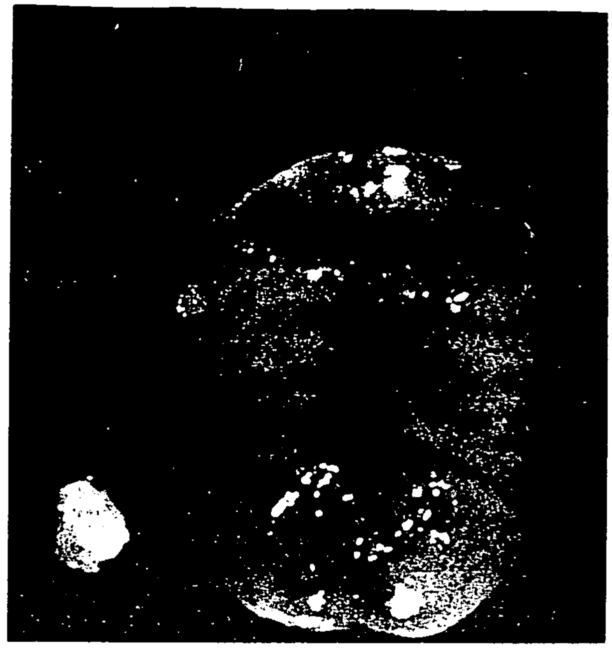
Fisher 344 male rats weighing 250-350 grams were anesthetized and placed in a stereotaxic apparatus. On day 0, we implanted $4x10^4$ 9L tumor cells in a volume of 50µL into the right cerebral hemisphere. 5 days later, the same stereotaxic coordinates were used to introduce either saline or $3x10^6$ STK producer line cells in 50µl directly into the growing tumor. 5 days later, the rats began treatment with GCV at 150mg/kg/dose twice daily. On the 5th day (10 days since inoculation of the tumor), the rats brains were examined for the extent of tumor growth. As seen in figure 6, the rats treated with the STK producer cell line and GCV are the only animals that experienced complete macroscopic elimination of the tumor (14 of 14 rats). Microscopic analysis revealed either no evidence of tumor (11 of 14 rats) or some residual, mostly necrotic, tumor in the tumor bed (3 of 14 rats). There was no evidence of vasculitis or destruction of normal tissues due to spread of the vector. This experiment further supports the data obtained with the G1NaSVBG vector, that this in vivo transduction method appears to be without significant side effects and has substantial efficacy.

jure 6.a. - Control rat brain. The tumor infiltrates and replaces the right frontal lobe.

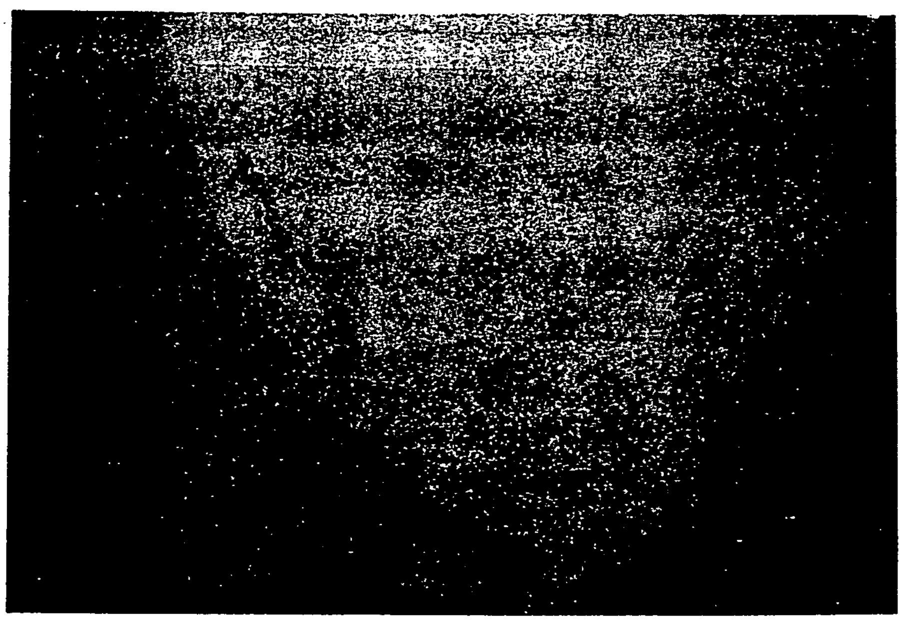


6.b. - Photomicrograph. Control rat brain; The highly malignant nature of the infiltrating 9L glioma is seen within the right frontal lobe. (H&E).

6.c. Rat brain after treatment with ganciclovir following transduction with the HS-tk gene. Note the absence of macroscopic tumor.



6.d. Photomicrograph. Section through the the cavity within the brain left after treatment with GCV following transduction with the HS-tk gene. Note the normal appearance of the adjacent brain and lack of cell infiltrate.



In subsequent series of experiments, we have evaluated the G1TKSVNa vector in the 9L brain tumor model and the various *in vitro* studies previously done with the G1NsCTK vector, using both producer and non-producer cells. Although no difference was found in our results between the different vectors (STK, G1NsCTK, G1TKSVNa), we believe that the G1TKSVNa vector is a better choice for use in a human clinical trial since it has an improved titer ($5x10^5$ versus $1x10^4$) and has further safety modifications to prevent the development of replication-competent virus compared to the other available vectors.

F. Bystander Effect.

One of the unique features of the tumor rejection in the HS-tk system in mice is the observation that not all the tumor's cells must contain the inserted gene in order to be killed upon ganciclovir challenge. In mice given a subcutaneous tumor in which 100% of the cells carry the HS-tk gene, complete tumor regressions are seen following GCV treatment. Interestingly, when tumors established from cell mixtures containing 50% HS-tk gene-modified cells mixed with 50% wild-type unmodified tumor cells were treated with GCV, almost all tumors (14/15) regressed. Even in situations where the mixed tumor contained 90% unmodified, wild-type tumor cells mixed with only 10% HS-tk modified tumor cells, complete regression of the cancer was observed with GCV treatment in 9 of 15 animals (see Table below).

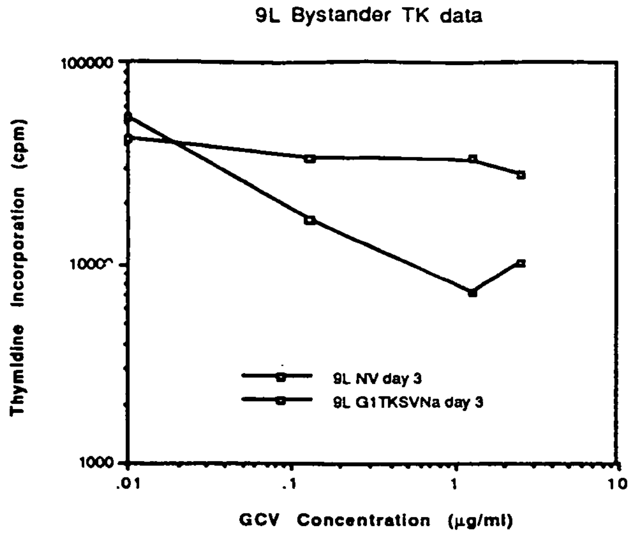
The effect of GCV on the incidence of tumor growth in mice injected with mixtures of various proportions of HS-tk gene-transduced and wild type tumor cells.

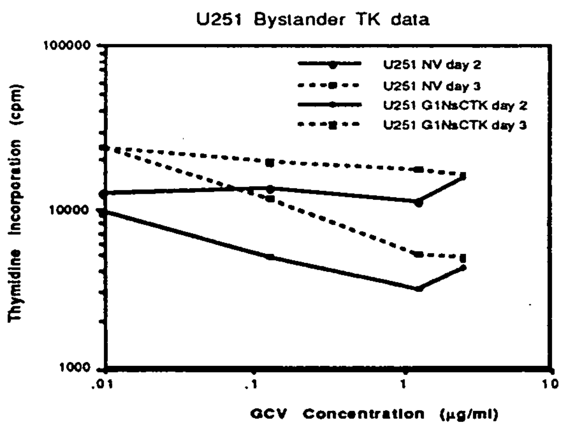
Tumor Mixture Recipient		Incidence of Palpable Tumors	
% wild type	% HS-tk tumor	2 weeks	5 weeks
0 %	100%	0/15	2/15
50%	50%	0/15	1/15
90%	10%	3/15	6/15
100%	0 %	12/15	15/15

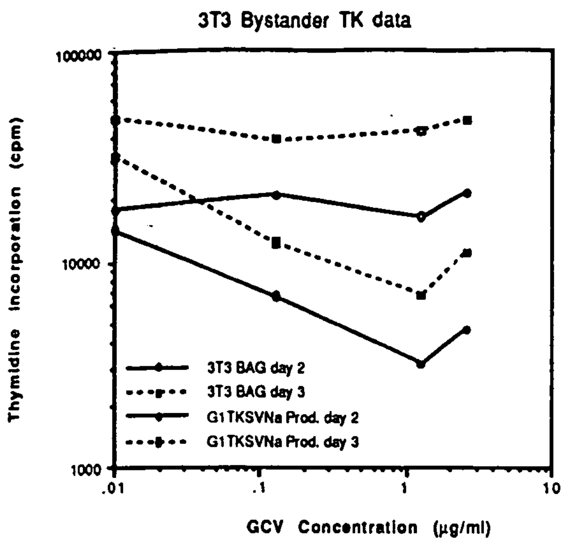
This phenomenon was reproduced in *in vitro* studies where we incubated various tumor cell lines (murine 205, rat 9L, human U251) with HS-tk-vector producer cells for 24 - 72 hours. Following exposure to GCV, the supernates were removed and transferred to tissue cultures of the wild-type tumors (not transduced with the HS-tk gene). Thymidine incorporation assays revealed that the supernates significantly inhibited thymidine incorporation in all tumor types (Figure 7). The mechanism of this "bystander tumor kill" is not yet completely understood. This may involve the production of toxic triphosphates by the interaction of thymidine kinase and GCV leading to inhibition of DNA synthesis and death of replicating cells. It does not seem to involve generalized non-specific cellular toxicity since the overlying skin and other tissues surrounding these HS-tk treated tumors was not grossly injured while the tumors expressing the genes and the admixed wild-type tumor cells were completely destroyed.

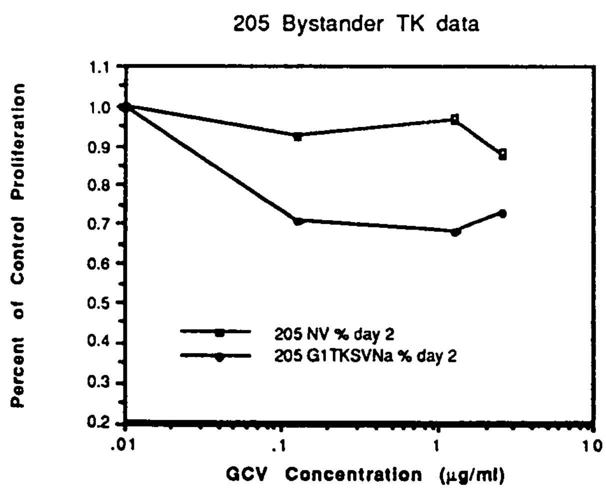
Both the 9L rat gliosarcoma and the human glioblastoma cell line U251 were also sensitive to this "Bystander" effect in an in vitro mixing experiment (see Appendix B; method by Hiroyuki Ishii). G1TkSvNa transduced and non-transduced tumor cells were mixed at different ratios in 96 well microtiter dishes. GCV was added to the wells and 24 hours later, the cultures were pulsed with tritiated thymidine. These bar graphs depict a greater decrease in proliferation than would be expected at a GCV level of 5.1 μg/ml in the medium (the numbers over the bars represent the percent of transduced tumor in the culture. This concentration is easily within the therapeutic range established in humans. Since it is unlikely that 100% of the tumor cells in the brains of our patients will be successfully gene-modified, this "bystander" effect is very important for the successful outcome of this treatment approach.

Figure 7:


9L Bystander TK data
100000
10000
1000
Thymidine Incorporation (cpm)
9L NV day 3
9L G1TKSVNa day 3
.01
.1
1
10
GCV Concentration (µg/ml)


U251 Bystander TK data
100000
10000
1000
Thymidine Incorporation (cpm)
U251 NV day 2
U251 NV day 3
U251 G1NaCTK day 2
U251 G1NaCTK day 3
.01
.1
1
10
GCV Concentration (µg/ml)


3T3 Bystander TK data
100000
10000
1000
Thymidine Incorporation (cpm)
.01
.1
1
10
GCV Concentration (µg/ml)
3T3 BAG day 2
3T3 BAG day 3
G1TKSVNa Prod. day 2
G1TKSVNa Prod. day 3


205 Bystander TK data
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
Percent of Control Proliferation
.01
.1
1
10
GCV Concentration (µg/ml)
205 NV % day 2
205 G1TKSVNa % day 2

Pre-Clinical Summary

The pre-clinical findings noted above suggest that the direct inoculation of a growing brain tumor with a HS-tk retroviral vector-producing cell line can mediate complete tumor rejection without the need for surgical excision, irradiation and/or traditional non-specific chemotherapy. In essence, this is an in vivo gene transfer methodology that selectively alters the sensitivity of a tumor cell to chemotherapy. Therefore, treatment with GCV does not result in widespread damage to the host immune system like many forms of chemotherapy.

Our animal studies have demonstrated no significant toxicity to the normal brain tissue or any of the proliferating non-CNS tissues evaluated suggesting that the implantation of vector producer cells in a brain tumor is not associated with non-specific systemic toxicity. Having no suitable brain tumor model in non-human primates, we propose a human clinical research protocol for the treatment of human brain tumors by the direct injection of G1TKSVNa vector-producing cells into primary and metastatic brain tumors.

III. Selection of Patients

All adults, greater than 18 years of age, with malignant brain tumors (primary and metastatic) who failed all standard therapy for their disease will be eligible to enter the study. Patients will be divided into two groups based on the surgical accessibility of their lesions as estimated from the pre-treatment radiological evaluation. These decisions will be made by the PI in accordance with the standards of care of neurosurgical practice.

Group 1. Patients in whom the lesion(s) is (are) surgically accessible with acceptable operative risk.
This group will provide necessary information regarding the efficiency of in-vivo transduction in the brain tumor after 5 days. In this group of patients the evaluation of the biological effect of tumor regression is limited and prolonged by the operative attempt at maximal tumor resection.
Group 2. Patients in whom the lesion(s) are surgically inaccessible or the operative risk is unacceptably high.
This group of patients will provide information regarding the effect of this treatment on

the tumor and will be measured by a change in tumor size. This change is completely dependent on the efficacy of transduction and is not biased by factors such as surgical debulking (as is the case in the surgically accessible group)

Three patients from either group who are considered to have an extremely poor prognosis will be included in the first treatment group for assessment of significant toxic side effects (which are not expected, based on the various animal studies).The study will continue as described if no such toxic effects will be encountered. An evaluation of toxicity report will be submitted to the Chairman, ICRS, NINDS, upon the completion of the study in these 3 patients.

Pregnancy testing will be performed on all eligible women. No pregnant woman will be entered into the study. HIV screening will be performed by the referring physician and patients with HIV infection will not be accepted for this study. Confirmatory non-serologic studies for HIV status will be performed at the NIH.

Patients admitted for study under this protocol will be registered with the principal investigator at Building 10, Room 5D37, National Institutes of Health, Bethesda, MD. 20892. [(301) 496-2239, 496-5728].

Exclusion Criteria: Patients with the following conditions will be excluded from the study:

1. Impairment of renal function. Patients must have a creatinine of less than 1.5 mg% or creatinine clearance greater than 80 ml/min/m^2.
2. Coagulopathy. Patients must have a normal coagulation profile as demonstrated by normal Prothrombin Time (PT) and activated Partial Thromboplastin Time (PTT).
3. Acute infection. Active infection is defined as any acute viral, bacterial or fungal infection which requires specific therapy.
4. Neurological deterioration. Patients with an increased ICP who require prompt reduction of ICP and have a surgically inaccessible lesion will be excluded.
5. HIV positive patients.
6. Pregnant patients.
7. Thrombocytopenia. Platelet count < 100,000 platelets /mm^3
8. Granulocytopenia. ANC < 1000/mm^3
9. Severe systemic diseases such as severe ischemic heart or lung disease considered to be associated with an unacceptable anesthetic/operative risk.
10. Patients with Karnofsky Score below 20 (see appendix) will be excluded from the study. (see appendix H).
11. Despite the requirement for a Durable Power of Attorney, as appears in the informed consent, patients who are unable to comprehend and sign the informed consent will be excluded from the study.

IV. Clinical Evaluation Before Treatment

A. Before a patient can be entered into this study, the NIH investigators must receive information from the referring physician(s) detailing the clinical history, general laboratory results, specific neurologic and radiologic evaluations, their diagnoses, and all previous therapies (Appendix A).

B. Data substantiating the histopathological diagnosis must be received for review prior to participation in the study. If no recent tissue diagnosis is available, the nature of the space occupying lesion (e.g. tumor vs. radiation necrosis) will be determined by a stereotaxic biopsy of the lesion prior to cell injection.

C. Pretreatment general physical examination and a comprehensive neurologic evaluation.

D. Renal, hepatic and hematological Evaluation including blood chemistries (uric acid, calcium, phosphorus, magnesium, osmolality, SGOT, SGPT, Alkaline Phosphatase, LDH, total bilirubin, BUN, creatinine, albumin, total protein, electrolytes, glucose), urinalysis with a 24 hr. creatinine clearance, PT, PTT, fibrinogen and a CBC with differential and platelets.

E. Radiological Evaluation. Each patient will undergo an MRI study of the brain without and with gadolinium enhancement. CT will be performed with contrast enhancement if appropriate. Preliminary decisions regarding the eligibility of the patient for either treatment group, and the injections and/or procedures needed for each patient will be decided based on these studies.

F. Immunologic evaluation.

1. *In vitro* lymphocyte proliferative responses to mitogens (OKT3, PHA), soluble antigens (Diphtheria, Tetanus, Candida albicans), alloantigen and murine antigens (PA317 cell).
2. Cellular phenotype of peripheral blood by dual label FACS analysis (CD3/HLA-DR, CD4/CD8, IL-2R)
3. Isohemagglutinins and quantitative immunoglobulins (A,G,M and E)
4. Serum for antibody to PA317 cells.
5. Determination of cytotoxic cell function (NK , LAK and CML as available)
6. Soluble IL-2R
7. DTH skin test panel (CMI Multitest or equivalent)
8. Non-serologic HIV test

V. Nature of Procedures or Therapeutic agents

(See schema in Appendix C)

A. Surgical Procedures

1. Surgically **accessible** group: Single lesion. Patients will undergo an MRI-guided stereotaxic injection of the HS-tk vector-producer cells into their brain tumor. The procedure will be performed under general anesthesia. Multiple injections will be performed into the tumor as determined by the size of the tumor. Each injection will be done using a volumetric pump over 10-30 minutes duration (based on the injected volume).

Seven days following the procedure, the patient will be taken to the operating room and under general anesthesia, a craniotomy will be performed and complete tumor removal will be attempted. The lining of the cavity will then be infiltrated at multiple sites with the HS-tk vector-producer cells. Ganciclovir will be administered starting on the fifth post-operative day for 14 days.

2. Surgically **accessible** group: Multiple lesions: Patients with multiple lesions (metastases) will be considered candidates for removal of multiple lesions according to the surgical feasibility of such procedures. In a selected group with multiple surgically accessible lesions, a staged surgical excision may be performed. Tumor transduction will be performed in two stages: the first will include stereotaxic injection of the lesions with the HS-tk-producer cells. Seven days later, the tumors will be removed via open surgery and intra-operative infiltration of the tumor lining will proceed as described in A.1. GCV will be administered as in A.1.

3. Surgically **inaccessible** tumors: Single lesion. Patients assessed as having a surgically inaccessible tumor will undergo an MRI-guided stereotaxic injection of the HS-tk vector-producer cells into their brain tumor. The procedure will be performed under general anesthesia. Multiple injections will be performed into each tumor as will be determined by the size of the tumor. Each injection will be done using a volumetric pump over 10-30 minutes duration (based on the injected volume). Ganciclovir administration will begin on the seventh day after the procedure for 14 days.

4. Surgically **inaccessible:** Multiple lesions: Patients harboring multiple surgically inaccessible lesions will have up to two stereotaxic injections performed in one single session. The number of procedures will be determined by the condition of the patient,

location(s) and the number of the lesions. Ganciclovir administration will begin on the seventh day after the procedure for 14 days.

B. Volume and number of injected cells:

Factors such as tumor size, location, and the preoperative neurological condition of the patient will determine the injectable volume. As a rule, the injection of a maximal number of vector producer cells in as small a volume as possible will be attempted. The final cell concentration will be adjusted to $\leq 1X10^8$ cells/ml.

C. Peri-operative medications:

1. Antibiotics: All patients will receive a single dose of IV 2g Ceftriaxone just prior to the surgical procedure.
2. Steroids: All patients will receive dexamethasone at 32 mg/day starting 7 days prior to the vector-producer cell injection and treatment will be continued until GCV is discontinued. Dexamethasone will then be tapered according to individual patient requirement. As experience is gained, the need for high dexamethasone dosing may be modified.
3. Mannitol: Will be administered during the surgical procedure at 1g/Kg and the dose repeated TID for 24 hours following the procedure.
4. Anticonvulsants: Anticonvulsive therapy will be administered according to the usual neurosurgical guidelines for such medications.
5. Analgesics: Pain medications will include Acetominophen 650-1000 mg Q 4 hours.

D. The G1TKSVNa Retroviral Vector

G1TKSVNa is a retroviral vector derived from the Moloney murine leukemia virus (MoMLV). This vector contains a herpes thymidine kinase (HS-tk) gene cDNA that is transcribed from the viral LTR and a bacterial neomycin resistance (NeoR) gene transcribed from an internal SV40 (simian virus 40) early promoter (LTR--HS-tk--SV--NeoR--LTR) in the G1 vector backbone (Genetic Therapy Inc., Gaithersburg, Md). This G1-based vector has been modified for increased safety by alteration of the gag start codon to a stop codon, and by elimination of viral sequences needed for the formation of the virus. This has been shown to minimize the potential for helper virus

production from producer cells which contain the vector. No replication-competent virus has been detected during long term culture or following administration of the vector to animals or humans.

The herpes simplex thymidine kinase (HS-tk) gene is a negative selectable marker or "suicide" gene. When a HS-tk transduced cell is exposed to ganciclovir (GCV), the GCV acts as a substrate for phosphorylation by HS-tk resulting in a triphosphate (TP) form of the drug. This phosphorylated form (GCV-TP) inhibits DNA polymerase and is incorporated into DNA resulting in an inability of the cell to proliferate. The end result is cell death for the HS-tk transduced cells (17). The NeoR gene is a positive selectable marker. The bacterial NeoR gene encodes for NPT II (neomycin phosphotransferase II), an enzyme that will protect G1TKSVNa expressing cells from the toxic effects of G418 (a neomycin analog). NeoR is widely used in our retroviral vectors and has been used in all human clinical trials to date without adverse effect. NPT-II inactivates the antibiotic Amikacin but does not inactivate other aminoglycoside antibiotics (such as gentamicin and tobramycin) (18). The introduction of the NeoR gene should not affect the clinical management of gram negative infections in the patients.

E. Preparation of the G1TKSVNa-Producer cell line

The vector construct was transfected into the PA317 (ATCC CRL 9078) packaging line cells. The generation of retroviral vectors from transinfected PA317 cells has been extensively tested *in vitro* and in human gene transfer/therapy experiments and in all cases has remained free of replication-competent retrovirus. (See I.B.3).

The transinfected G1TKSVNa cells were selected in G418 and cloned. Clone 90 has produced the highest NeoR and HS-tk titer ($5x10^5$ cfu/ml for both) and this clone has been submitted for FDA approved testing for use in our clinical studies. Prior to the use of the G1TKSVNa-producer cell lines for injection into humans, the cells will be required to meet FDA specifications already in use for other clinical human gene therapy experiments in progress.

F. Growth of the G1TKSVNa Producer cell line for Clinical Use

The producer cell lines will be grown in either monolayer in flasks or in an artificial capillary system (ACS).

1. The producer cells are maintained in complete medium which contains Dulbecco's modified MEM with 4.5 gm glucose/liter with L-glutamine (DMEM), 10% FCS, 2 mM L–glutamine, 100 U/ml penicillin G sodium and 100 mcg/ml streptomycin sulfate.
2. Monolayer Cultures

   The monolayer culture will be grown in T–175 flasks at $37^{o}C$ with 5% CO_2. When the cells are >90% confluent, the cells will be trypsinized and split 10:1 in fresh complete medium. A confluent T-175 flask generally contains $1\text{-}1.5X10^7$ cells.
3. ACS Cultures

   The producer cells grown in five T-175 flasks are trypsinized, washed in complete medium and resuspended in 100 ml of media. The cells are then injected into a autoclaved, CELLMAX™ 100 bioreactor (Cellco Advanced Bioreactors, Inc.; Kensington, MD) and the bioreactor is placed in a $37^{o}C$, 5% CO_2 incubator. Perfusate is pumped continuously through the 8000 hollow fibers to maintain proper nutrition and eliminate waste products of cellular metabolism as previously described (19). In brief, complete medium is pumped at a flow rate of 10–300 ml/minute, increasing as the number of cells increase. Glucose measurements will taken periodically from the perfusate to aid in assessing the need for media change. The 100 ml cartridge can reach near tissue density, containing approximately 10^{10} cells (equivalent to the number of cells contained in 1000 confluent flasks). We have grown G1-based vectors in the ACS on multiple occasions without evidence of development of aberrant growth or replication-competent retrovirus.

G. Testing and Harvest of the Producer cell lines for Clinical Use

1. 48 hours prior to clinical use
   a. Sterility cultures
   b. Endotoxin level
2. On the day of cell harvest for clinical use:
   a. An aliquot of supernate will sent for a STAT gram stain and culture.
   b. Supernate will be at saved at $-70^{o}C$ for viral titer and tests for replication

competent virus (S+/L-).

c. The cells will be trypsinized, harvested into 50 ml conical tubes (Falcon), washed three times in normal saline and counted by trypan dye exclusion.
d. An aliquot of cells will be cryopreserved for DNA studies.
e. The final cell concentration will be adjusted to no greater than $1x10^8$.

VI. Potential Hazards of the Implantation of G1TKSVNa Producer Cell lines into Human Brain Tumors

A. Potential surgical complications.

Infection: Patients with malignant brain tumors have a significant predisposition to a variety of superimposed infections secondary to a state of immune suppression as previously described. The probability of post operative wound infection is also increased due to previous radiation damage to the scalp following radiotherapy. Antibiotic therapy will be given as a prophylaxis prior to the surgical interventions and specific infections will be treated as needed.

Increased ICP: Patients with an intracranial space occupying lesion often present with symptoms attributable to increased intracranial pressure (ICP). All the patients in this study will be treated prophylactically with high dose dexamethasone before the surgical procedures. Additional measures (Mannitol) will be given in the peri-operative period.

Steroid complications: The long term effects of steroids are well documented. However, most of the patients who will enroll in our study will require steroids for control of increased ICP regardless of the proposed treatment. Inducing a reduction in tumor size may actually allow the reduction of the steroid dose in some patients.

Chemical meningitis: Despite the lack of a meningeal reaction in our model laboratory animals, meningitis or meningitis-like symptoms may develop secondary to spillage of the vector-producer cells into the subarachnoid space. Such symptoms are expected to be self-limiting and ameliorated with symptomatic care (analgesics). However, such a reaction may be severe and could produce severe permanent neurological deficits or death. When indicated, CSF sampling may be required to rule

out infection.

Severe edema: Edema secondary to necrosis and breakdown of the transduced tumor cells may occur. The magnitude of such edema, if it occurs at all, is unkown. As a precaution, all patients will be treated with high-dose steroids and peri-operative mannitol as preventive/therapeutic measures. If edema around the tumor is not responsive to therapy, it can result in permanent, significant neurological deficits or death.

Surgical Procedures: The surgical procedures (both stereotaxic and open surgery) carry a risk for loss of neurological function, non-neurological complications and death. The risk depends on the preoperative condition of the patient, size and location of the tumor, and associated diseases (e.g. Ischemic heart disease, renal failure, COPD etc.). The risk for an individual patient will be determined prior to the decision on the appropriate line of therapy and will be discussed with the patient prior to surgery.

B. The development of replication-competent retrovirus.

This has been discussed (Section V.D.).

C. Prolonged survival of the PA317 murine cells in vivo.

The PA317 cells are murine cells that survive 7-10 days in syngeneic (H-2^b) mice and 7-14 days in rats bearing brain tumors. Xenogeneic antigens on transplanted tissue induce immune rejection in immunocompetent humans. Therefore, the immune response of the patient will be expected to eliminate all of the injected producer line cells. The time to rejection is difficult to estimate since the brain is a partially immunologically privileged site, patients with brain tumors are immunosuppressed due to the presence of the tumor, and the patients will be receiving high-doses of exogenous corticosteroid therapy. Lastly, on day 7 we will initiate treatment with GCV that will destroy any remaining PA317 cells.

D. Dissemination of G1TKSVNa.

Our MoMLV amphotropic retroviral vectors are directly inactivated by human complement (without antibody). Therefore, escape of free vector into the cerebral spinal fluid or the vascular space should result in immediate inactivation. The

gene transfer in this direct injection system is most likely due to the intimate contact of the tumor and producer cell lines. Any vector particles that are released in the area of injection will be quickly bound by the thousands of amphotropic vector receptors on each tumor cell and other host cells. Even if all of the vector particles produced were able to escape direct transfer into adjacent tumor cells and cross the blood-brain barrier, the number of vector particles relative to the number of receptors in any organ would still be very small suggesting a very small risk of injury to proliferating cells in any non-CNS organ. Any direct transfer into neurons by cell-to-cell contact will not result in HS-tk gene integration and therefore, should not pose a risk for their destruction with GCV treatment.

E. <u>Transduction of surrounding brain tissue.</u>

There is no evidence in our animal model that transduction of surrounding normal brain tissue is likely to be a problem (Section II.A.2.). However, if the HS-tk gene will be introduced into a large number of normal dividing cells within the CNS (such as endothelial cells and astroglial cells), vasculitis like symptoms (headaches, convulsions, bleeding) may develop. Such changes however will be localized to the immediate vicinity of the tumor as had been shown in our experiments (II.B.2) and in fact may even enhance tumor eradiacation by increasing the exposure to the immune system.

F. <u>Insertional Mutagenesis</u>

Retroviral vector DNA is inserted randomly into the genome of proliferating cells. The random nature of this integration allows for the potential of an untoward insertional event. If the insertion disrupts a gene essential for maintaining cell function, that particular cell will die. Since the gene transfer will occur most predominantly in tumor cells, if the vector insertion site results in the death of a few tumor cells without GCV, that should not pose a problem.

The risk of oncogeneic transformation with these retroviral vectors cannot be accurately estimated since that has never been a documented occurrence in animals or man. While this is a real risk, this risk must be very low, especially in this protocol, where all vector containing cells will be killed by GCV. In our 189.8 months of cumulative patient observation in the human gene transfer clinical protocol and the 89.9 years of cumulative observation of primates (some severely immunosuppressed

and intentionally exposed to large amounts of infectious replication-competent retrovirus), no untoward effects of retroviral exposure or retroviral-mediated gene transfer have been observed (20) (Appendix E). Based upon available data, the risk of death secondary to their tumor far exceeds any risk of insertional mutagenesis.

G. **Ganciclovir sodium (GCV; Cytovene®)**

The GCV used in this trial will be purchased from Syntex corporation (Palo Alto, CA). GCV is an FDA approved drug for the treatment of cytomegalovirus (CMV) retinitis in immunocompromised individuals. The drug is administered by intravenous infusion over 1 hour at a dose of 5 mg/kg of body weight twice daily for 14-21 days. FDA approved prescribing guidelines (where applicable), administration procedures, drug interactions, and patient monitoring recommendations will be followed for the use of this drug. The Cytovene® product monograph is included in appendix F.

There is no information regarding the use of GCV for the treatment of humans as a method to destroy herpes TK gene transduced human cells. GCV does cross the blood-brain barrier. The cerebral spinal fluid (CSF)/plasma ratios has been estimated in 3 patients at various time intervals with ratios ranging from 0.24 to 0.7 (0.31-0.68 μg/ml in the CSF and 0.44-2.20 μg/ml in the plasma). Peak plasma levels have been documented to reach 9μg/ml. These CSF and plasma levels are expected to be within the range of GCV levels needed to kill the HS-tk transduced cells based on *in vitro* studies (0.5μg/ml will prevent growth of HS-tk-transduced tumor cells). If the patient has evidence of renal impairment, the dose will be adjusted as suggested in the GCV monograph.

The most common side effects are granulocytopenia (absolute neutrophil count (ANC) of ≤1000 cells/mm^3) in 40% of patients and thrombocytopenia (≤ 50,000 platelets/mm^3) in 20%. This data was collected in immunosuppressed, CMV infected AIDS patients, who may have been more susceptible to marrow suppression than our patient population, due to additional opportunistic infections and concomitant drug therapy. The actual risk to our patient population is unknown. Each patient will be closely monitored for the development of granulocytopenia and thrombocytopenia. The development of an ANC of <500 cells/mm^3 or a platelet count of <25,000 platelets/mm^3 will require a dose interruption until the ANC is ≥750 cells/mm^3 platelet

count is $\geq$40,000 platelets/mm^3.

Other side effects occurring in approximately 2% of patients include anemia, fever, rash and abnormal liver function. If possible, autologous blood will be obtained and stored prior to treatment for transfusion should significant anemia develop secondary to GCV therapy or surgical blood loss. Fever and chills will be treated with acetominophen (650 mg every 4 hours) and rash with diphenhydramine (50mg every 6 hours). See appendix G for complete toxicity criteria.

There are a number of other possible toxicities related to GCV administration (e.g. the possibility of temporary or permanent inhibition of spermatogenesis) discussed in the monograph (Appendix F; page 38). These toxicities have generally occurred in immunosuppressed, ill individuals receiving long term administration. Our treatment protocol will use the known tolerated dose (10mg/kg/day) in non-infected patients for only 14 days. These differences in patient population and treatment duration should minimize the likelihood of the development of potential toxicities cited.

H. <u>Reporting of Adverse Reactions</u>

Adverse drug reactions (ADRs) to the IND Drug will be reported promptly to the Investigational Drug Branch (IDB), phone (301) 496-7957. ADR reports are required even if there is only a suspicion of a drug effect. Previously unknown Grade 1,2 and 3 reactions will be reported to the NCI in writing using the "NCI Adverse Reactions Form for Investigational Agents" within 10 working days. Grade 4 (life-threatening) reactions and patient deaths while on treatment will be reported to the NCI by phone within 24 hours. A written report will follow within 10 working days.

Written reports will be sent to:
Investigational Drug Branch
Cancer Therapy Evaluation Program
P.O.Box 30012
Bethesda, MD 20824

VII. Evaluation and Follow Up.

A. Evaluation during the treatment period:

1. Before GCV treatment. Patients will be evaluated daily throughout the study. For the first 48 hours following the surgical procedure, they will be under continuous monitoring in the Surgical ICU.

The neurological exam will be recorded daily. MRI scan of the brain will be obtained on the first and fifth post-operative day depending on the clinical status.

2. During GCV treatment. Patients will return to the Surgical ICU for GCV therapy, for a minimum of 48 hours. A Neurological exam will be performed and recorded twice a day. An MRI study, with and without gadolinium, will be performed at least weekly beginning on the second day of GCV therapy. Creatinine clearance tests, liver function tests, CBC with differential count, coagulation studies and blood chemistry will be closely monitored.

B. Evaluation following the treatment period.

1. Follow up. Patients will be prepared for discharge upon completion of the GCV course of treatment. A complete neurological and physical examination will be recorded prior to discharge. Patients will be seen as outpatients at 2 weeks intervals for the first 2 months and on a monthly basis for one year. Neurological status will be recorded at each follow up visit as well as a gadolinium enhanced MRI, blood chemistry and CBC. The number of visits after the first year will depend on the status of the tumor.

2. Yearly follow-up laboratory evaluation.

Patients will be requested to be followed once a year for the rest of their life for retroviral gene transfer safety monitoring.

a. CBC with Differential count
b. Repeat Immunologic evaluation.
c. Serum for antibody to PA317 cells.
d. PCR on mononuclear cell DNA for vector sequences
e. Western analysis of serum for antibody to retroviral antigens
f. If at any time a new malignancy develops, we shall attempt to obtain involved tissue for analysis for the vector DNA.

It is understood that the performance of an individual study or test as specified in this protocol is subject to factors such as patient compliance, scheduling difficulties, equipment malfunction, or the clinical judgment of the principal investigator or patient care physician, and that a test therefore may not be done in an individual instance with no violation of the protocol. However, any systematic modification of the original protocol in this regard, whether related to patient safety or not, will be submitted to the IRB for approval.

3. Tumor specimen processing and evaluation: Previously injected tumors removed during open surgery will be subjected to routine histopathological evaluation, staining for the presence of murine cells and DNA analysis for NeoR and Env (envelope) sequences. The relative proportion of NeoR to Env will provide a semiquantitative measure of the degree of tumor transduction.

4.Autopsy: An attempt will be made to perform a complete autopsy on any patient who dies during the study. Whenever possible, tissues from the brain, tumor(s), bone marrow, will be evaluated for the presence of the G1TKSVNa vector by PCR. Whenever the autopsy is performed outside the NIH, an attempt will be made to obtain the pathological slides of the CNS for review at the NIH. A tentative agreement to autopsy is included as part of the consent form.

C. Criteria for Response

1) **Non responders**: No observable decrease in the size or progression of the tumor by MRI scan.
2) **Partial responders**: >50% decrease in tumor volume by MRI at any time point .
3) **Complete responder:**
   a. No remaining tumor on MRI at 3 months for the surgically accessible groups
   b. No remaining tumor on MRI at 4 weeks for the surgically inaccessible groups.

D. Off-Study Criteria

a. The development of any grade 3 or 4 toxicity that is not easily correctable. It should be noted that the assessment of neurological toxicity (Neurosensory, neuromotor, neurocortical, neurocerebellar, neuro-mood, neuro-headache, etc. as appears in Appendix G - Toxicity Criteria) relates to the relative changes from the pre-treatment neurological status.

b. Upon request of the participating patient or the person with the power of attorney.

VIII. References

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